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Molecular characteristics of extracellular matrix-associated glycosaminoglycans in endothelium[1]

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ABSTRACT The extracellular matrix-associated sulfated glycosaminoglycans synthesized by bovine corneal endothelial cell clones consisted of 80% heparan sulfate and 20% chondroitin sulfate. The M_r distribution of heparan sulfate and chondroitin sulfate fractions were determined by high speed gel permeation chromatography. The average M_r of matrix-associated heparan sulfate of endothelial cell clones 1,2 and 3 were respectively 48 000, 41000 and 38 000. A size analysis of the degradation products after acidic nitrous acid deamination indicated that the degree of *N*-sulfation of heparan sulfate in clone 1 endothelial cells were relatively high. Heparan sulfate from all three clones was degraded by incubating purified heparan sulfate fractions with a metastatic B 16 melanoma cell lysate containing heparanase (heparan sulfate specific endo-β-glucuronidase). The average size of the heparan sulfate degradation products was M_r 10 000.

KEY WORDS endothelium; extracellular matrix; glycosaminoglycans; neoplasm invasiveness; neoplasm metastasis; proteoglycans

Endothelium synthesizes and secretes GAG, a portion of which is deposited into the subendothelial ECM as PG[1]. The subendothelial ECM-PG contain GAG such as HS, which may be involved in controling cell-ECM interactions during tumor cell metastatic invasion of the basal lamina[2,3], neurite outgrowth and regeneration of blood vessels after vascular injury. In many systems GAG in the interstitial spaces between cells as well as those at the cell surface appear to be important in the control of cell adhesion, proliferation and morphogenesis. In this report the major ECM-associated sulfated GAG of cloned BCE were determined to be HS which was degraded by the enzyme secreted from metastatic melanoma cells.

MATERIALS AND METHODS

Cell culture BCE were kindly provided by Dr Gospodarowicz (UCSF) and cloned BCE (C 1.1, C 1.2 and C 1.3) were obtained by the limiting dilution technique and maintained as previously[4]. All cells were grown, maintained and used at low passages ($p<10$).

Preparation of [^{35}S]GAG BCE-C 1.1, -C 1.2 and -C 1.3 were subcultured at a

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ABBREVIATIONS BCE = bovine corneal endothelium; BCE-Cl. 1, -Cl. 2 or -Cl. 3 = bovine corneal endothelium clone 1,2 or 3; CS = chondroitin sulfate; DS = dermatan sulfate; ECM = extracellular matrix; FGF = fibroblast growth factor; GAG = glycosaminoglycans; HS = heparan sulfate; PG = proteoglycans

density 1.5×10^5/10 cm dish or 1.5×10^4/3.5 cm dish for 12 d to allow them to reach confluency. At this time the medium was replaced with sulfate-free complete medium containing 5% fetal bovine serum and 0.74 GBq/ml [^{35}S] sulfuric acid (New England Nuclear, Boston MA) for 48 h.

Isolation of [^{35}S] GAG from ECM [*sulfate*-^{35}S] ECM on plastic substratum was digested with 2 ml of Pronase (Calbiochem-Behring, La Jolla CA) solution (1 mg/ml) for 10 h at 37℃. The supernatant was transferred into plastic tubes and incubated for 12 h. After centrifugation at $9000\times g$ the supernatant was lyophilized, dissolved in 0.4 *M* pyridine-sodium acetate buffer, pH 5.0, and applied to a 1×18 cm column of Sephacryl S-200 (Pharmacia, Piscatway NJ). The ^{35}S-labeled material was further fractionated by anion exchange chromatography[5] using a 1×18 cm column of DEAE-sephacryl (Pharmacia) with a linear concentration gradient of 0.05 *M*/0.05 *M* to 3.1 *M*/3.1 *M* pyridineformate buffer, pH 5.0[5].

Chemical and enzymatic treatments of GAG For chondroitinase-ABC (Miles) treatment, 0.8 ml of an aqueous solution of GAG was mixed with a 0.2 ml solution of the enzyme (2 IU/ml) and heated to 100℃ for 5 min, and then centrifuged at $9000\times g$ for 5 min. Undegraded GAG were recovered from the supernatant by gel filtration on Bio-Gel P-6. Nitrous acid deamination was performed[6]. Homogenates of B 16-B 15 b murine melanoma cells were prepared as the source of heparanase[3].

Analytical procedures Agarose gel electrophoresis was performed in 0.7% agarose in 50 m*M* 1,3-diaminopropane-acetate buffer pH 9.0[7]. After gel electrophoresis, gels were washed, dried and processed for autoradiography with Kodak Z-Omat AR-5 X-ray film. Densitometric scanning (550 nm) of the autoradiograms was performed in a Beckman model DU-8 spectrophotometer. High-speed gel permeation chromatography of sulfated GAG was performed on 2 sequential 0.7 oligosaccharides in the M_r range 600-60 000.

RESULTS

Fractionation of sulfated GAG The major radioactive peak corresponding to approximate M_r 40 000-50 000 were estimated from the elution of standard GAG, when the ECM-GAG was separated by gel filtration on Sephacryl S-200; and the separation was not so clear (Fig 1 Left). After having applied to a DEAE-cellulose ion-exchang chromatography column, a major radioactive peak eluted at the position coincided with that of the standard bovine lung HS and was tentatively identified as HS (Fig 1 Right). A small shoulder was seen following the major radioactive component. But complete separation of this latter component was not achieved in this chromatography system. From the elution position of standard human

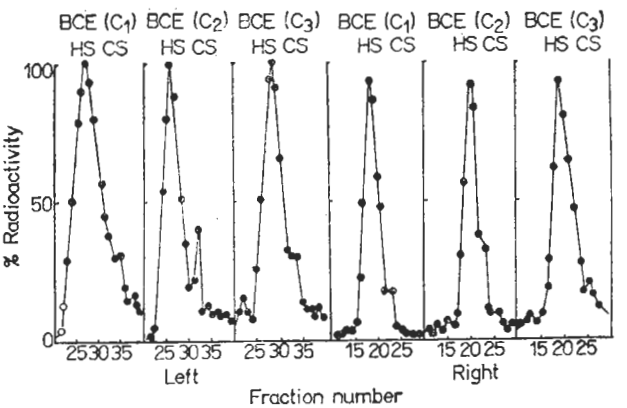

Fig 1. Left: Gel filtration on Sephacryl S-200 of high molecular weight material derived from the pronase digested fraction of the ECM of BCE clones 1-3. Right: Purification of GAG by DEAE-Sephacel chromatography. The columns were standardized with HA, DS, C_4S and C_6S.

umbilical cord hyaluronic acid (Miles), the sulfated GAG should be separated from contaminated hyaluronic acid at this step. All radioactive peaks should be processed further.

Analysis of GAG by agarose gel electrophoresis Autoradiogragraphs of ECM-associated [sulfate-^{35}S]ECM synthesized by BCE-C 1.1, -C 1.2,-C 1.3, and separated by agarose gel electrophoresis showed 2 descrete bands. The migration of the minor band coincided with CS (Fig 2). In this

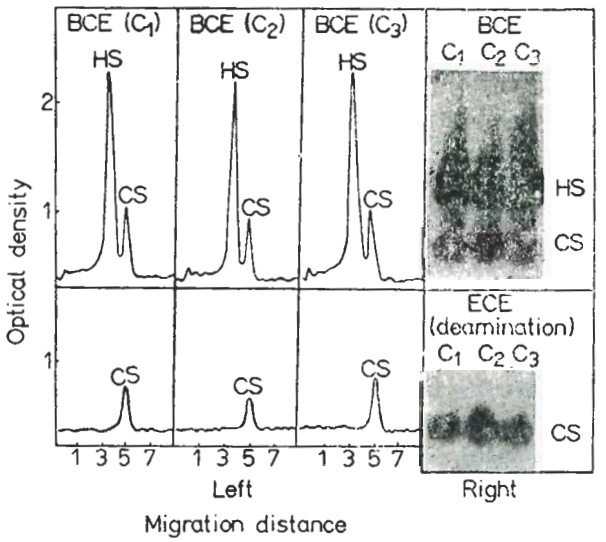

Fig 2. Autoradiographs of ECM-associated GAG synthesized by BCE clone 1-3 and separated by agarose gel electrophoresis before and after deamination by densitometric scanning of the autoradiogram (Left) and the pictures of electrophoresis (Right).

system we could not determine the type of CS. When the sulfated GAG preparations were previously treated with nitrous acid, the major band disappeared, indicating that it was HS. However, the minor bands remained unchanged after such a treatment. The relative proportions of HS to CS in the ECM-associated sulfated GAG sysnthesized by BCE-C 1.1, -C 1.2 and -C 1.3 were estimated as 77.2/22.8, 79.2/20.8 and 79.5/20.5.

Molecular weight distribution of GAG The ECM-associated HS synthesized by BCE-C 1.1, -C 1.2 and -C 1.3 were M_r 48 000, 41 000 and 38 000, after separation from agarose gel electrophoresis and analysis in a highspeed gel permeation chromatography. The peak of [^{35}S] CS appeared to be broader than that of HS, and each [^{35}S] CS fraction eluted as a single peak. The position of the peak of CS indicated that the average M_r was in the range of 20 000 to 45 000 (Fig 3 Left)

Further characterization of HS The elution profiles of the Bio-Gel P-6 purified and chondroitinase ABC treatmented [^{35}S] HS upon high speed gel permeation chromatography were similar to the patterns obtained with [^{35}S]HS preparations after agarose gel electrophoresis. Nitrous acid deamination was carried out on purified [^{35}S]HS under conditions that resulted in cleavage of *N*-sulfonyl or *N*-free glucosaminyl linkages intact, and the reaction products of ECM-associated HS derived from BCE-C 1.1 cells eluted at a position of M_r 1000, the approximate size of sulfated tetrasaccharide ftagments analyzed using the gel permeation system (Fig 3

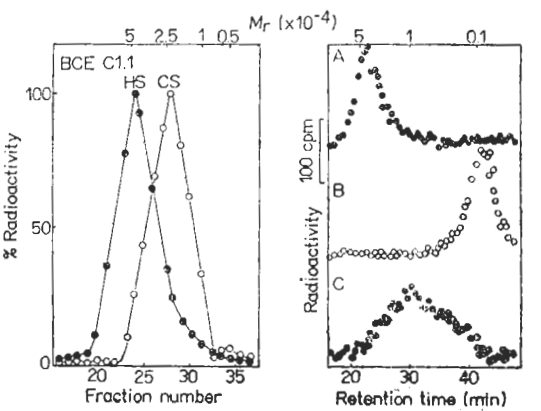

Fig 3. Analysis of [^{35}S]HS and [^{35}S]CS of BCE-C 1.1 by high-speed gel permeation chromatography (Left). Analysis of [^{35}S]HS after chondroitinase ABC treatment. A) Untreated HS; B) HS treated with nitrous acid; C) HS treated for 6 h with B16 melanoma heparanase. Arrows indicated the elution positions of standard glycans ($M_r \times 10^{-3}$)(Right).

Right). Absence of larger fragments was probably due to the relatively even distribution of glucosamine without *N*-acetyl groups along the HS chain.

Purified [^{35}S]HS were tested for their susceptibility to cleavage by tumor cell heparanase (HS-specific endo-β-glucuronidase) of B 16 melanoma cells. After incubating the ECM-associated [^{35}S]HS synthesized by BCE-C 1.1 cells with a lysate of B 16-B 15 b melanoma cells for 3-6 h in the presence of D-saccharic acid 1,4-lactone, a strong inhibitor of glucuronidase. The resultant HS fragments eluted at a retention time corresponding to M_r 10000 (Fig 3 Right). The position of the major peak did not change upon further incubation with the B 16 heparanase.

DISCUSSION

Although sulfated GAG molucules such as HS are distributed throughout mammalian tissues and are molecularly diverse in terms of their size and degree of sulfation[9], studies have not been performed correlating the molecular diversity of HS to specific cellular interactions. In our studies [*sulfate*-^{35}S]ECM were obtained after BCE cells reached confluency. This condition was used because ECM is synthesized at only low levels before cell confluency[10], and also because the BCE cells appeared to be more radiosensitive during logarithmic growth. Therefore, we cannot eliminate the Possibility that BCE sulfated GAG in the ECM produced before the cells reached confluency may differ from those synthesized at confluency. Cell growth rates and densities have been found to affect the profiles of GAG synthesis in other systems[11]. However, the radioactive materials in our purified HS and CS are probably representation of the major portion of the sulfated GAG pool, since on the gel permeation system the radioactive peaks coincided with the peaks detected by monitoring carbonyl absorption at 210 nm.

The degradation products of ECM-associated sulfated GAG synthesized by BCE-C 1.1 after acidic nitrous acid deamination consisted of sulfated tetrasaccharides. This strongly suggested that the distribution of non-*N*-acetylated (presumably *N*-sulfated) glucosaminyl residues was relatively even along the polymer, and most of every other glucosamine molecule was non-*N*-acetylated. The relatively high degree of *N*-sulfation in HS produced by endothelium was also suggested by the results of Gamse and co-workers[1]. Although refered to as a heparin-like molecule, its sensitivity to B 16 melanoma heparanase indicated that the ECM-associated HS synthesized by BCE-C 1.1 was not heparin-like since the melanoma enzyme did not degrade heparin[3]. Interestingly, the size of the major degradation products of HS from bovine lung[3] or PYS-2 carcinoma cells[8] were smaller than that from endothelium after incubation with tumor cell heparanase.

Endothelial ECM-associated GAG have been implicated in various types of tumor cell interactions, such as those taking place during blood-borne tumor cell-blood vessel binding and tumor cell penetration leading to metastasis formation[12]. Subendothelial ECM appears to serve as a preferential adhesion site for metastatic tumor cells[13], although the molecules responsible for malignant cell-binding to ECM have only been partially eludidated[13]. When metastatic tumor cells interact with radioactively labeled subendothelial ECM glycoproteins such as fibronectin, laminin, collagen and PG, such as HS, speific degradation occurs[2,38,12]. We have found that the ability of mouse B 16 melanoma sublines degraded purified bovine lung HS, correlated with the organ colonizing potential of these cells[3]. Using a rapid ana-

lytical system to determine the sizes of GAG and their degradation products we have characterized the fragments produced by incubation of melanoma cells with purified bovine lung HS, and demonstrated that B 16 cells possess a HS-specific degradative enzyme (heparanase) which is an endo-β-glucuronidase. Incubation of the B 16 heparanase with HS from BCE-C 1.1, a degraded products of M_r 10000 was obtained.

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内皮细胞外基质氨基聚糖的分子性质

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提要　在体外培养的内皮细胞外基质中，硫酸化的氨基聚糖主要由 80%左右的硫酸乙酰肝素及 20%的硫酸软骨素所组成。硫酸乙酰肝素的 *N*-硫酸化程度很高，其分子量从 38000 到 48000。从转移性黑色素瘤 B 16 细胞中纯化得到的硫酸乙酰肝素专一性的 β-葡萄糖醛酸内酶可降介从内皮细胞分离而得到的硫酸乙酰肝素，降介产物的平均分子量为 10 000。这与肿瘤细胞的浸润与转移密切相关。

关键词　内皮细胞；　细胞外基质；　氨基聚糖；　肿瘤浸润；　肿瘤转移；　蛋白聚糖

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